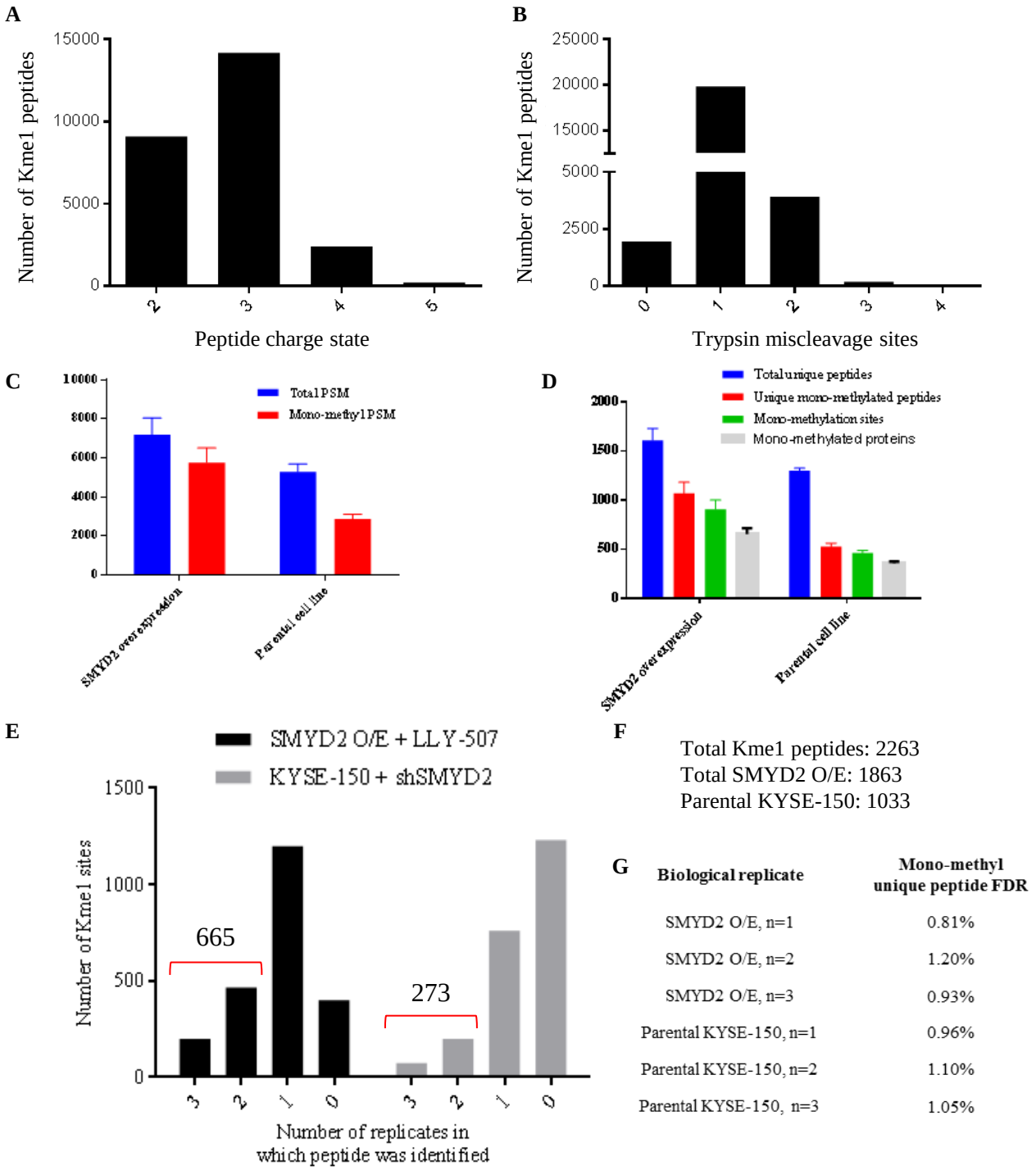
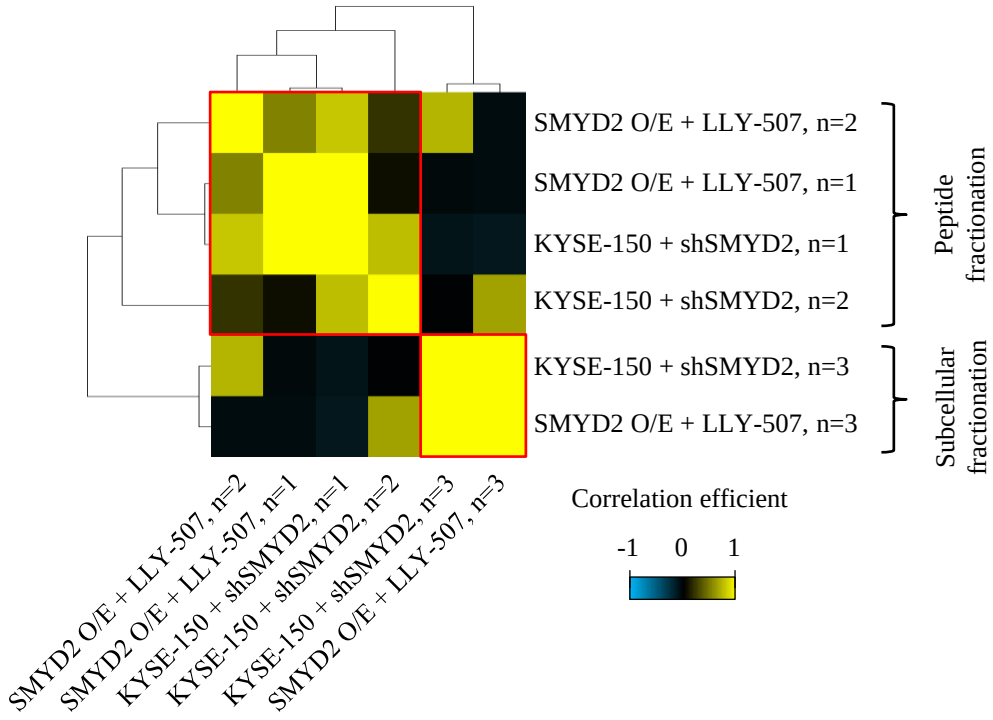


Biological replicate	Mono-methyl unique peptide FDR
SMYD2 O/E, n=1	0.81%
SMYD2 O/E, n=2	1.20%
SMYD2 O/E, n=3	0.93%
Parental KYSE-150, n=1	0.96%
Parental KYSE-150, n=2	1.10%
Parental KYSE-150, n=3	1.05%

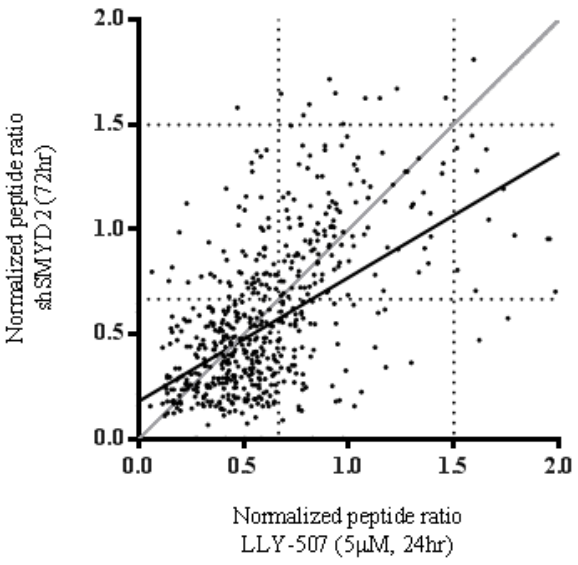
Figure S1: Summary of SILAC LC-MS datasets: (A) Distribution of charge states for all peptides identified in the biological triplicate SILAC-based LC-MS datasets. (B) The number of trypsin miscleavage sites per peptide identified (C) The number of peptide spectral matches (PSM) and mono-methyl PSM in the dataset. (D) The averaged number of unmodified and mono-methylated unique peptides as well as mono-methylated proteins and Kme1 sites from 3 biological replicate experiments. (E) The reproducibility of Kme1 site identification among the two cell conditions (parental and SMYD2 overexpression cell lines) among biological triplicate assays. The 665 and 273 Kme1 sites used for analysis and relative quantification are indicated. (F) The number of mono-methylated (Kme1) peptides identified in total and in the two experimental conditions. (G) The false-discovery rate (FDR) of mono-methylated peptides in the indicated biological replicates.

Figure S1

A



B

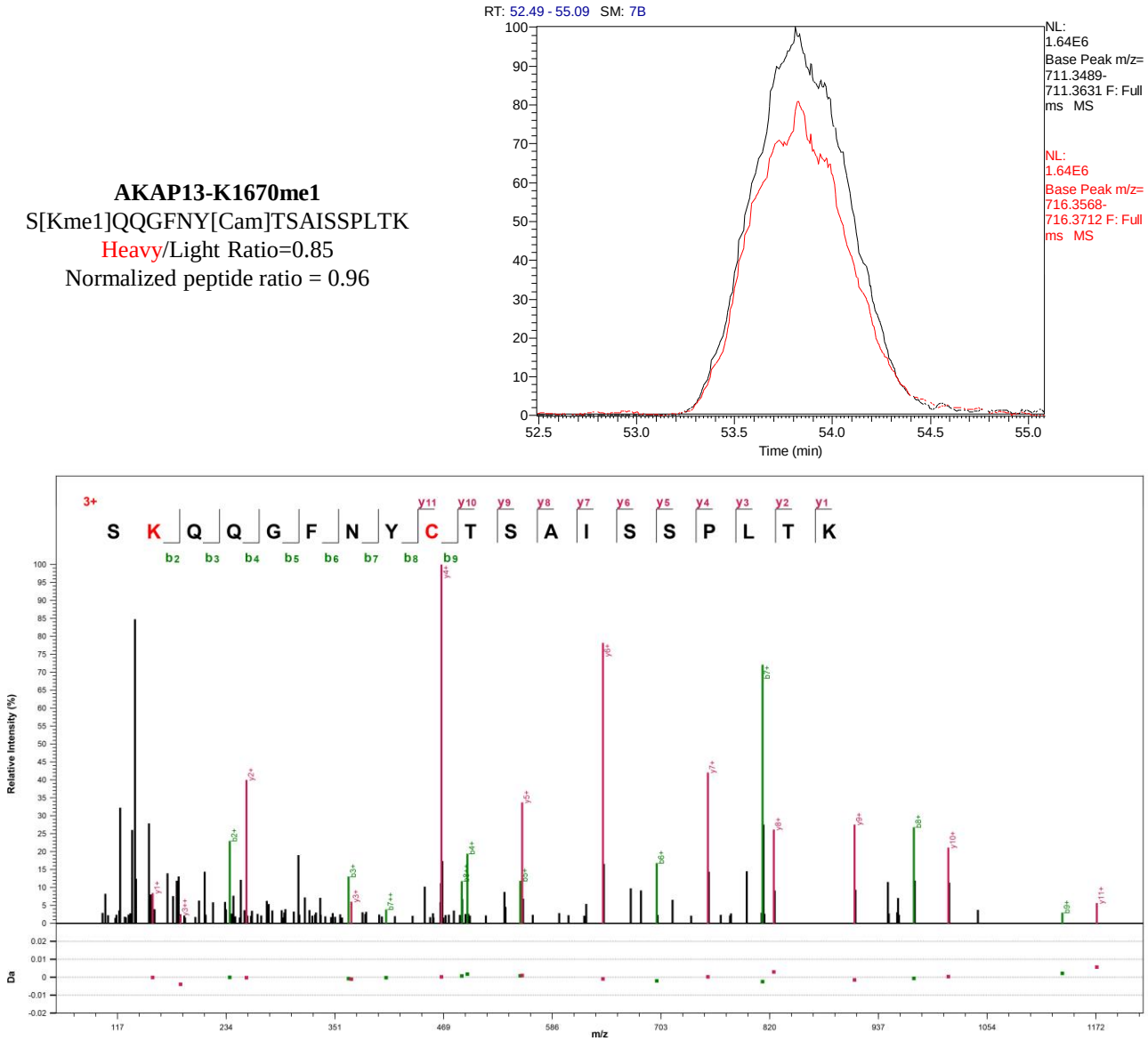


Best-fit values	
Slope	0.5932 ± 0.01150
Y-intercept when X=0.0	0.1788 ± 1.058
X-intercept when Y=0.0	-0.3014
1/slope	1.686
95% Confidence Intervals	
Slope	0.5707 to 0.6158
Y-intercept when X=0.0	-1.894 to 2.252
X-intercept when Y=0.0	-3.809 to 3.186
Goodness of Fit	
R square	0.8083
Sy.x	26.54

Figure S2: Reproducibility of SILAC-based quantification between biological experiment: (A) Pair-wise cross-correlation scores were calculated by average linkage for the indicated biological replicates. Replicates were grouped by hierarchal clustering. (B) Scatterplot of individual Kme1 peptides and the averaged normalized peptide ratio in the shSMYD2 and SMYD2 overexpression + LLY-507 treatment datasets. The black line is the line of best fit (R^2 = 0.8083), and the grey line represents the main diagonal.

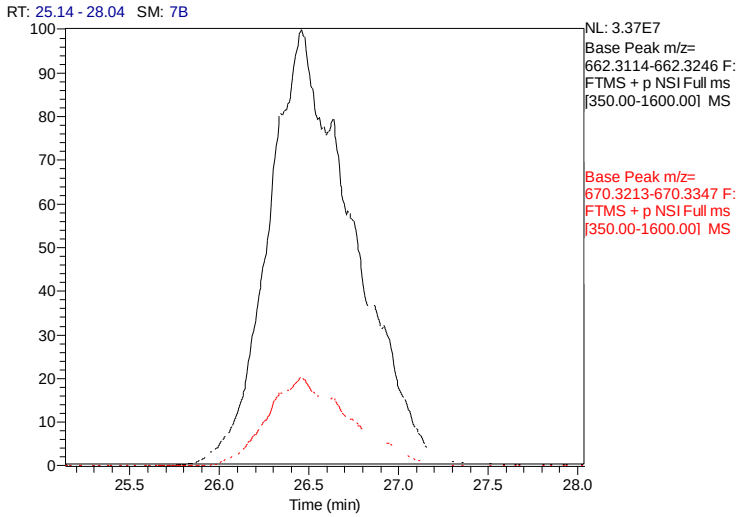
Figure S2

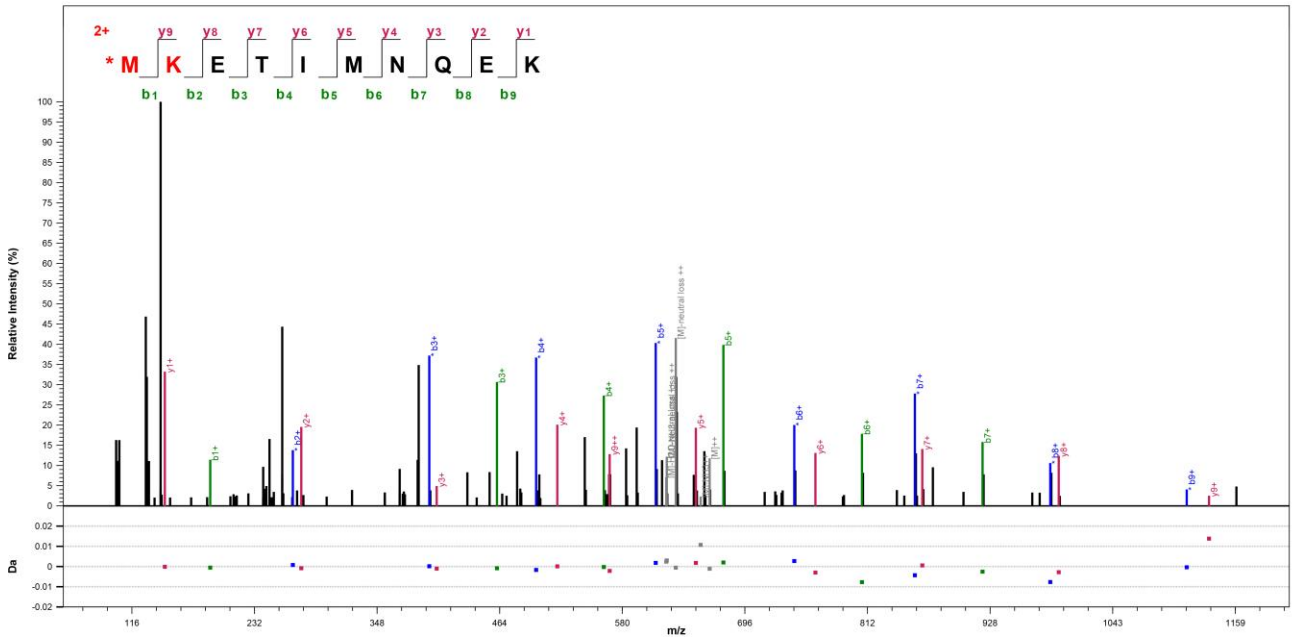
AKAP13-K1670me1
S[Kme1]QQGFNY[Cam]TSAISSPLTK
Heavy/Light Ratio=0.85
Normalized peptide ratio = 0.96



<u>Figure S3: SILAC quantification for AKAP13-K1670me1.</u> The top panel is the extracted ion chromatograms (XICs) for the base peak of the AKAP13-K1670me1 peptides derived from the mixed protein lysates of shSMYD2 (red, heavy-labelled) and shCON (black, light-labelled). As expected, the two peptides have identical retention times. The ratio of the heavy and light peptide base peak MS1 intensities are indicated in the top left, as is the normalized peptide ratio. The bottom panel contains the MS2 spectrum of the labelled AKAP13-K1670me1 peptide with the identified fragment ions labelled.

BTF3-K2me1
ac-[Mox][Kme1]ETIMNQEK
Heavy/Light Ratio=0.19
Normalized peptide ratio = 0.22

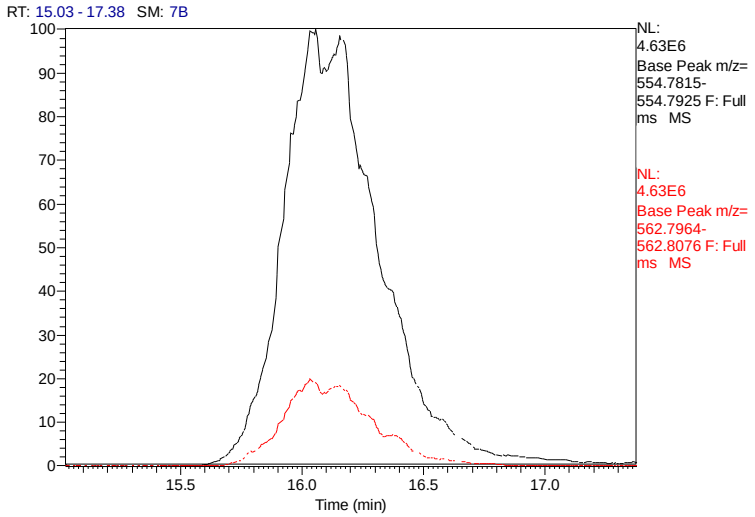


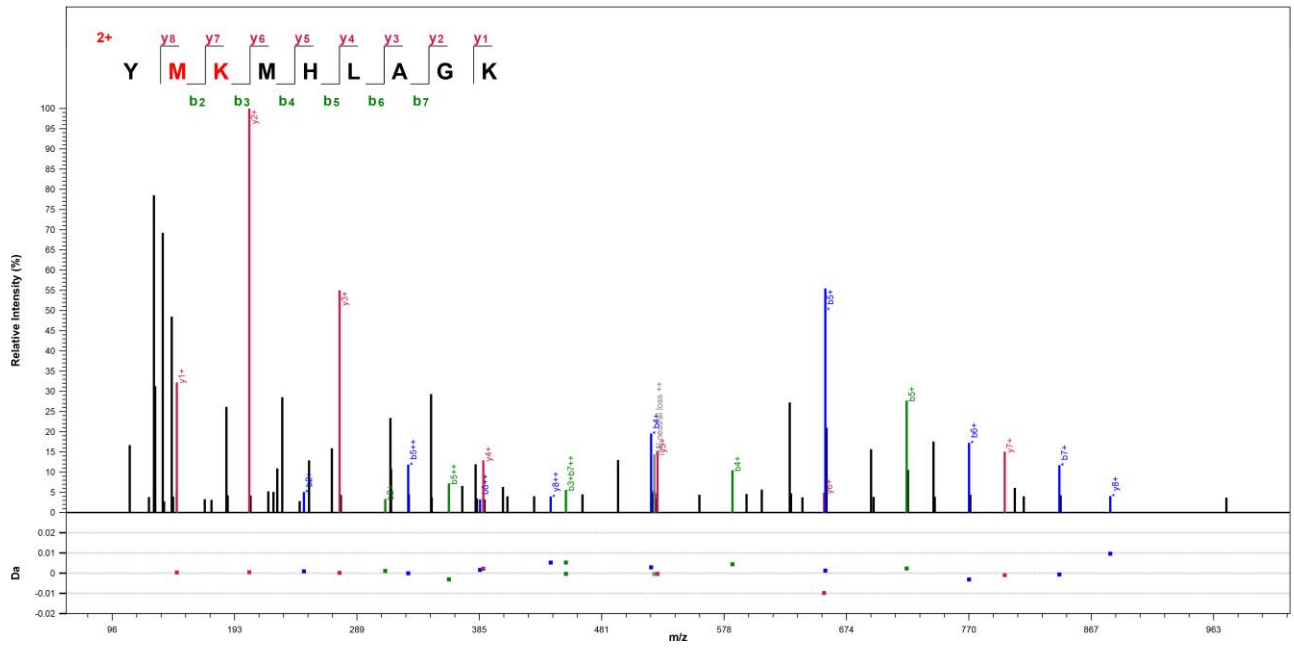


Blue and * b ions are b ions lost SOCH4 from oxidized Met.

Figure S4: SILAC peptides for BTF3-K2me1. The top panel is the extracted ion chromatograms (XICs) for the base peak of the BTF3-K2me1 peptides derived from the mixed protein lysates of shSMYD2 (red, heavy-labelled) and shCON (black, light-labelled). As expected, the two peptides have identical retention times. The ratio of the heavy and light peptide base peak MS1 intensities are indicated in the top left, as is the normalized peptide ratio. The bottom panel contains the MS2 spectrum of the unlabelled BTF3-K2me1 peptide with the identified fragment ions labelled.

PDAP1-K126me1
Y[Mox][Kme1]MHLAG
Heavy/Light Ratio=0.18
Normalized peptide ratio = 0.20





Blue and * b ions are b ions lost SOCH4 from oxidized Met.

Figure S5: SILAC peptides for PDAP1-K126me1. The top panel is the extracted ion chromatograms (XICs) for the base peak of the PDAP1-K126me1 peptides derived from the mixed protein lysates of shSMYD2 (red, heavy-labelled) and shCON (black, light-labelled). As expected, the two peptides have identical retention times. The ratio of the heavy and light peptide base peak MS1 intensities are indicated in the top left, as is the normalized peptide ratio. The bottom panel contains the MS2 spectrum of the unlabelled PDAP1-K126me1 peptide with the identified fragment ions labelled.

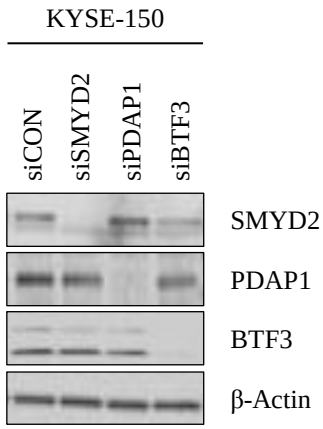


Figure S6: Effect of SMYD2 knockdown on BTF3 and PDAP1 protein levels. (**A**) Western blot assays monitoring the expression of the indicated proteins in KYSE-150 cells in response to siRNA-mediated knockdown of SMYD2 (siSMYD2), PDAP1 (siPDAP1) or BTF3 (siBTF3) or control siRNA (siCON) for 72 hr.

Figure S6

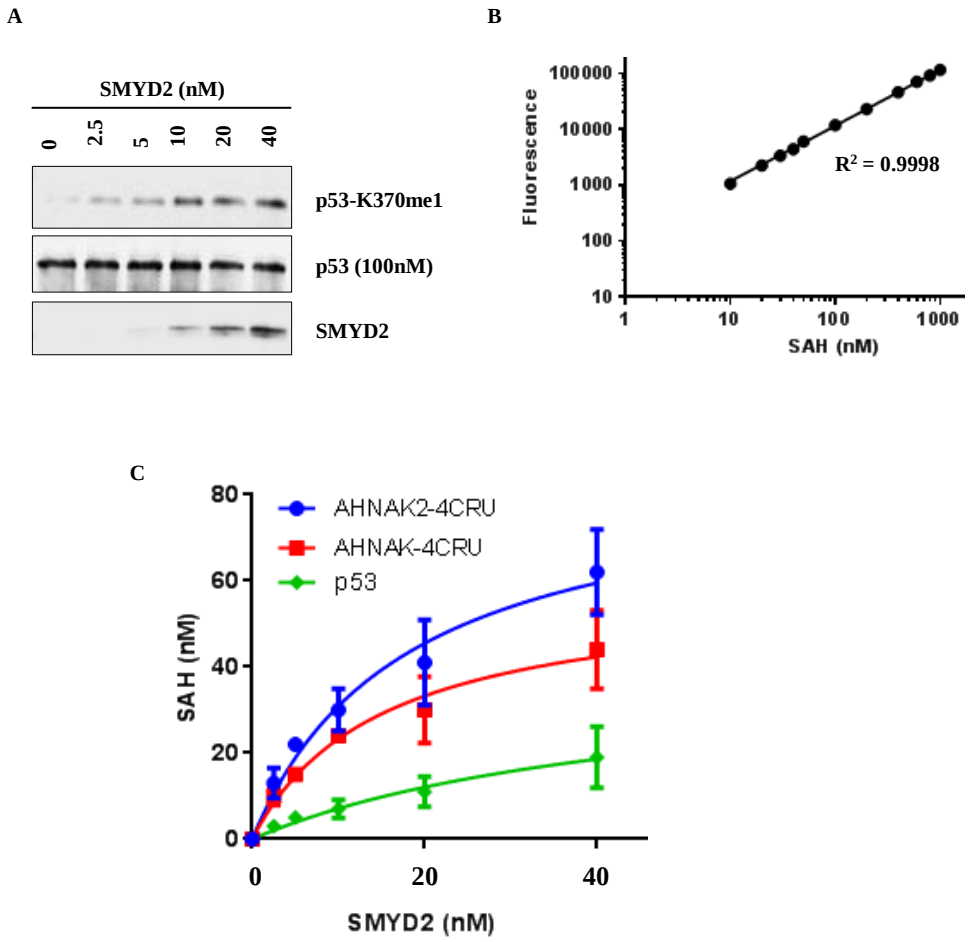


Figure S7. SAH ELISA data for *in vitro* SMYD2 methylation assays

(**A**) Western blot monitoring the levels of p53-K370me1, total p53 and SMYD2 following incubation of the indicated concentration of SMYD2 with 100nM p53. (**B**) Standard curve for fluorescence readings from replicate SAH ELISA assays using the indicated concentration of SAH. (**C**) SAH ELISA data for 100nM recombinant AHNAK-4CRU, AHNAK2-CRU, and p53 and the indicated concentrations of SMYD2.

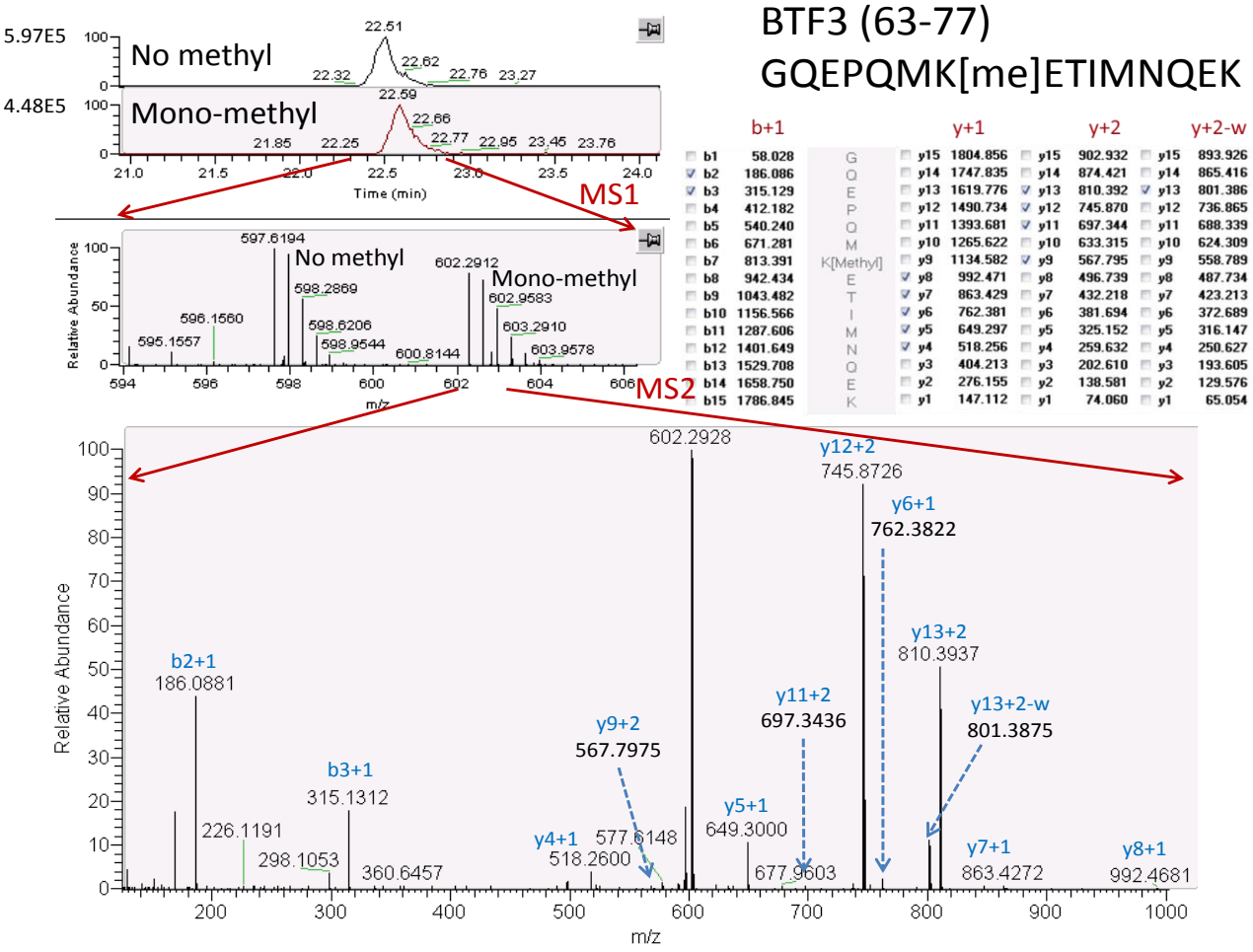


Figure S8. Peptide from his-tagged recombinant full-length BTF3 protein (amino acids 63-77) with mono-methylation on K69 (equivalent to K2 in the 18kD isoform or K46 in the 23kD isoform). Both un-methylated and mono-methylated peptides were observed with the same amino acid sequence due to miscleavage at K69. Note that Lys followed by Glu often shows high level of miscleavage by trypsin in many other proteins. The two peptides have similar retention time, as shown on top-left panel for extracted ion chromatograms (XICs) and MS1 spectrum. Annotated MS2 spectrum, represented in the bottom part, is for mono-methylated peptide only. The expected fragment m/z values are shown in the upper right panel. Those fragments with check marks are those observed from the MS2 spectrum, with "-w" indicating loss of water.

Figure S8

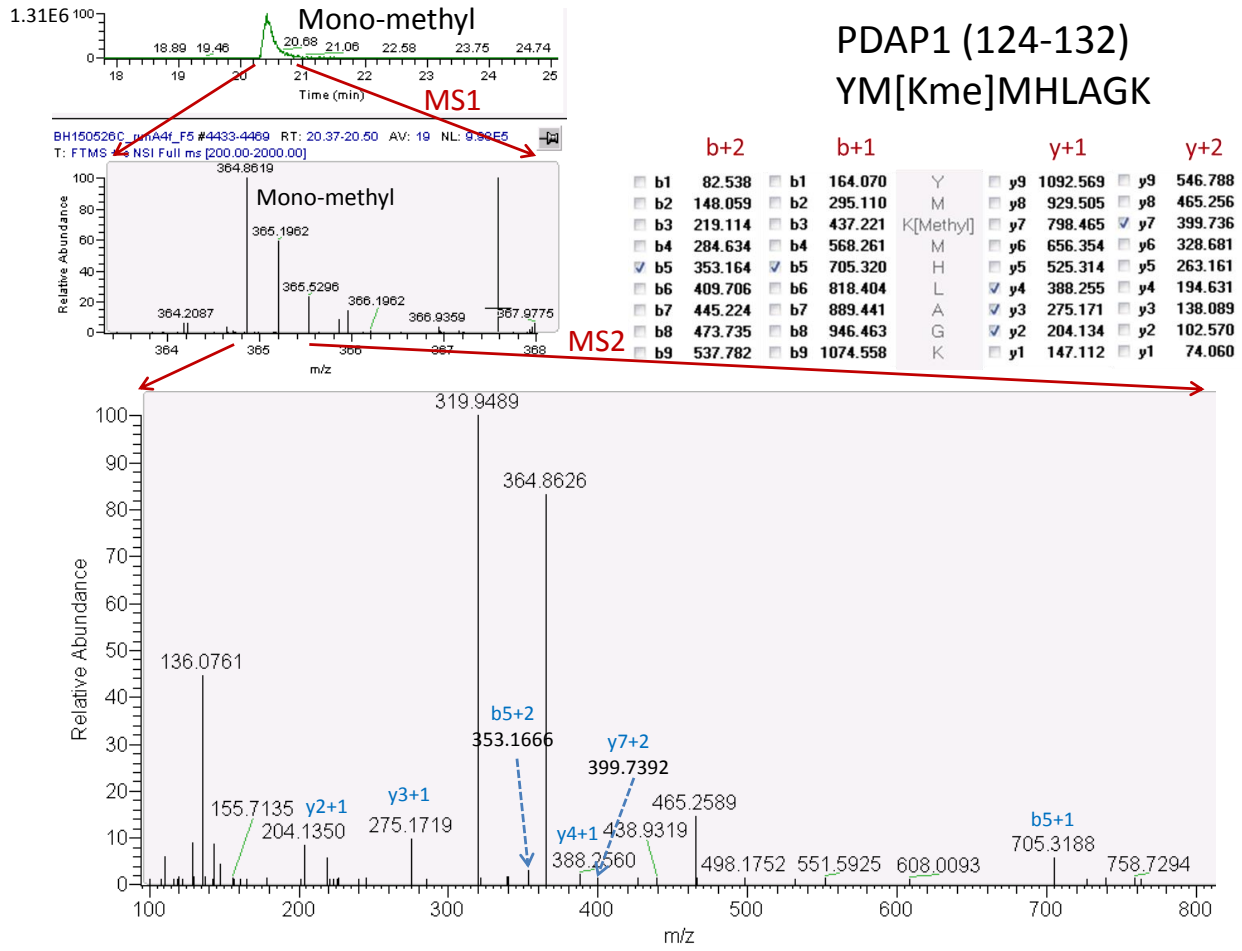
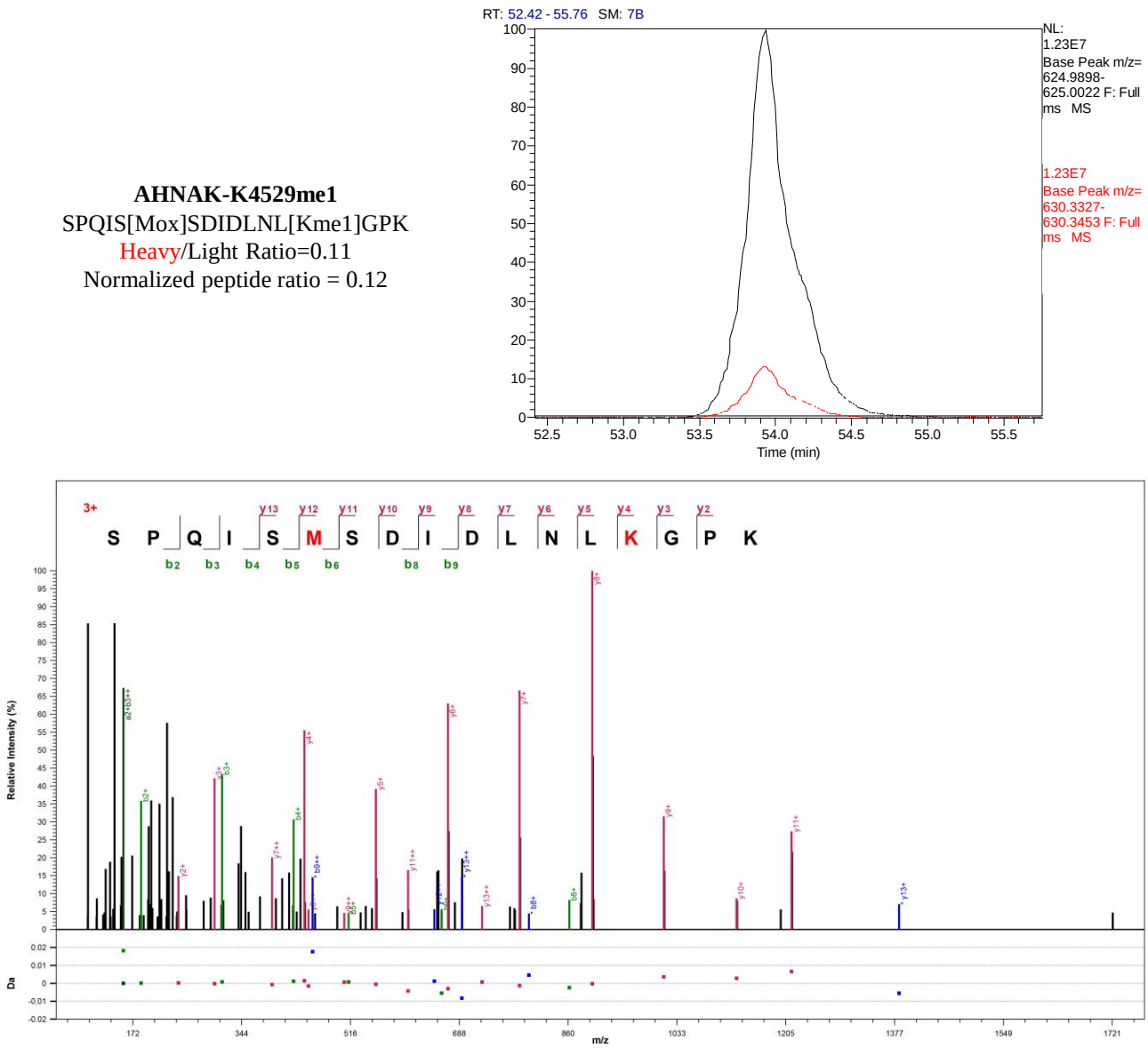


Figure S9. Peptide from his-tagged recombinant full-length PDAP1 protein (amino acids 124-132) with mono-methylation on K126. Only the methylated peptide was observed. Un-methylated peptide would be cleaved by trypsin at K126 and was not observed. The trypsin-cleaved un-methylated peptide, MHLAGK, was not observed, probably because the peptide was too hydrophilic to be captured reliably on the C18 column in typical reverse phased HPLC conditions. The top-left panel shows the extracted ion chromatogram (XIC) of the mono-isotopic m/z value and the MS1 spectrum under the XIC peak. The bottom panel shows the MS2 spectrum of the mono-methylated peptide. The expected fragment m/z values are shown in the upper right panel. Check marks indicate fragments that were observed in the MS2 spectrum.

AHNAK-K4529me1
SPQIS[Mox]SDIDLNL[Kme1]GPK
Heavy/Light Ratio=0.11
Normalized peptide ratio = 0.12

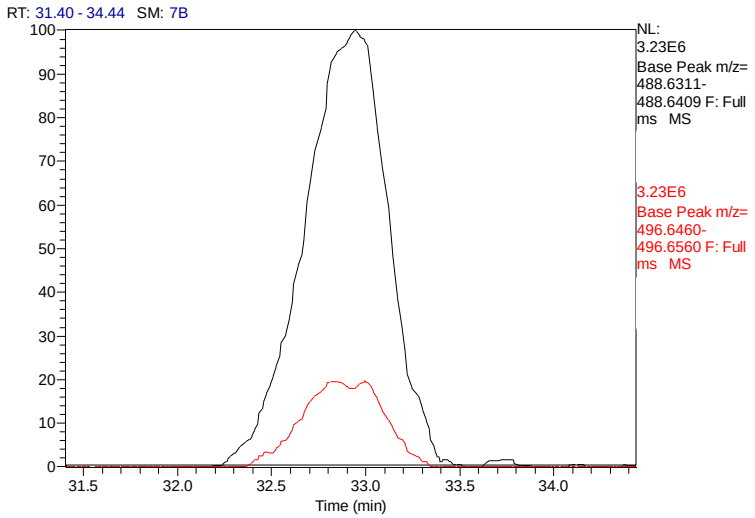


Blue and * b ions are b ions lost SOCH4 from oxidized Met.

Figure S10: SILAC peptides for AHNAK-K4529me1. The top panel is the extracted ion chromatograms (XICs) for the base peak of the AHNAK-K4529me1 peptides derived from the mixed protein lysates of shSMYD2 (red, heavy-labelled) and shCON (black, light-labelled). As expected, the two peptides have identical retention times. The ratio of the heavy and light peptide base peak MS1 intensities are indicated in the top left, as is the normalized peptide ratio. The bottom panel contains the MS2 spectrum of the unlabelled AHNAK-K4529me1 peptide with the identified fragment ions labelled.

Figure S10

AHNAK2-K1107me1/K1114me1
VAL[Kme1]GPQVDV[Kme1]GPK
Heavy/Light Ratio=0.20
Normalized peptide ratio = 0.22



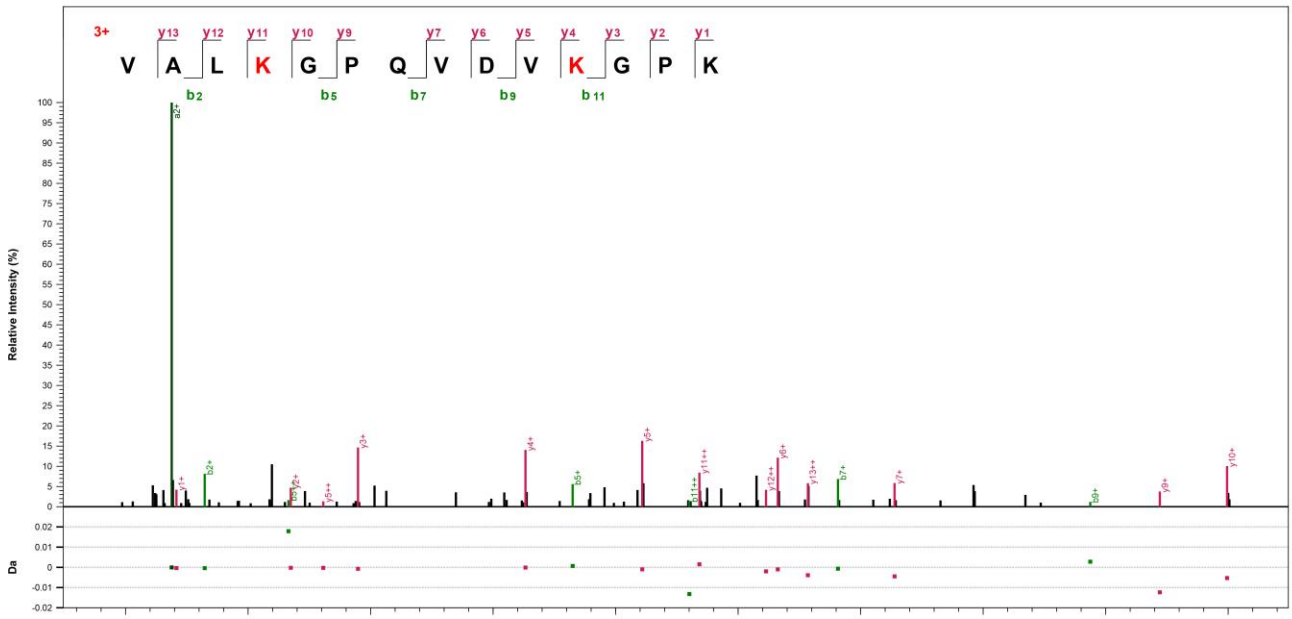


Figure S11: SILAC peptides for AHNAK2-K1107me1/K114me1. The top panel is the extracted ion chromatograms (XICs) for the base peak of the AHNAK2-K1107me1/K1114me1 peptides derived from the mixed protein lysates of shSMYD2 (red, heavy-labelled) and shCON (black, light-labelled). As expected, the two peptides have identical retention times. The ratio of the heavy and light peptide base peak MS1 intensities are indicated in the top left, as is the normalized peptide ratio. The bottom panel contains the MS2 spectrum of the unlabelled AHNAK2-K1107me1/K1114me1 peptide with the identified fragment ions labelled.

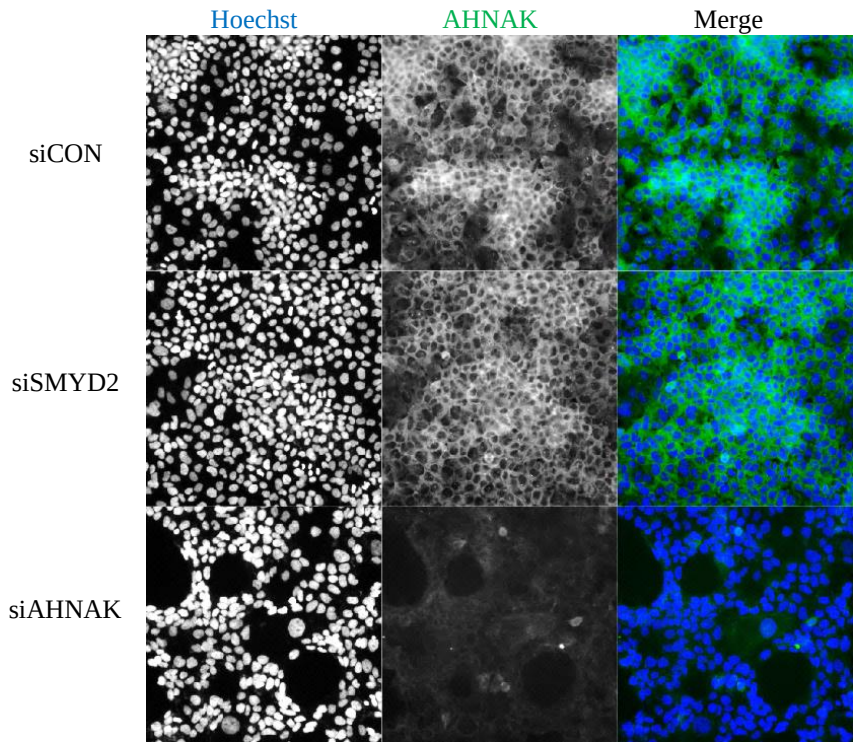


Figure S12. Protein expression of SMYD2 and SMYD2 substrates in mammalian cell lines. Immunofluorescence images monitoring the expression of AHNAK in KYSE-150 cells in response to siSMYD2, siAHNAK or siCON treatment for 48 hours.

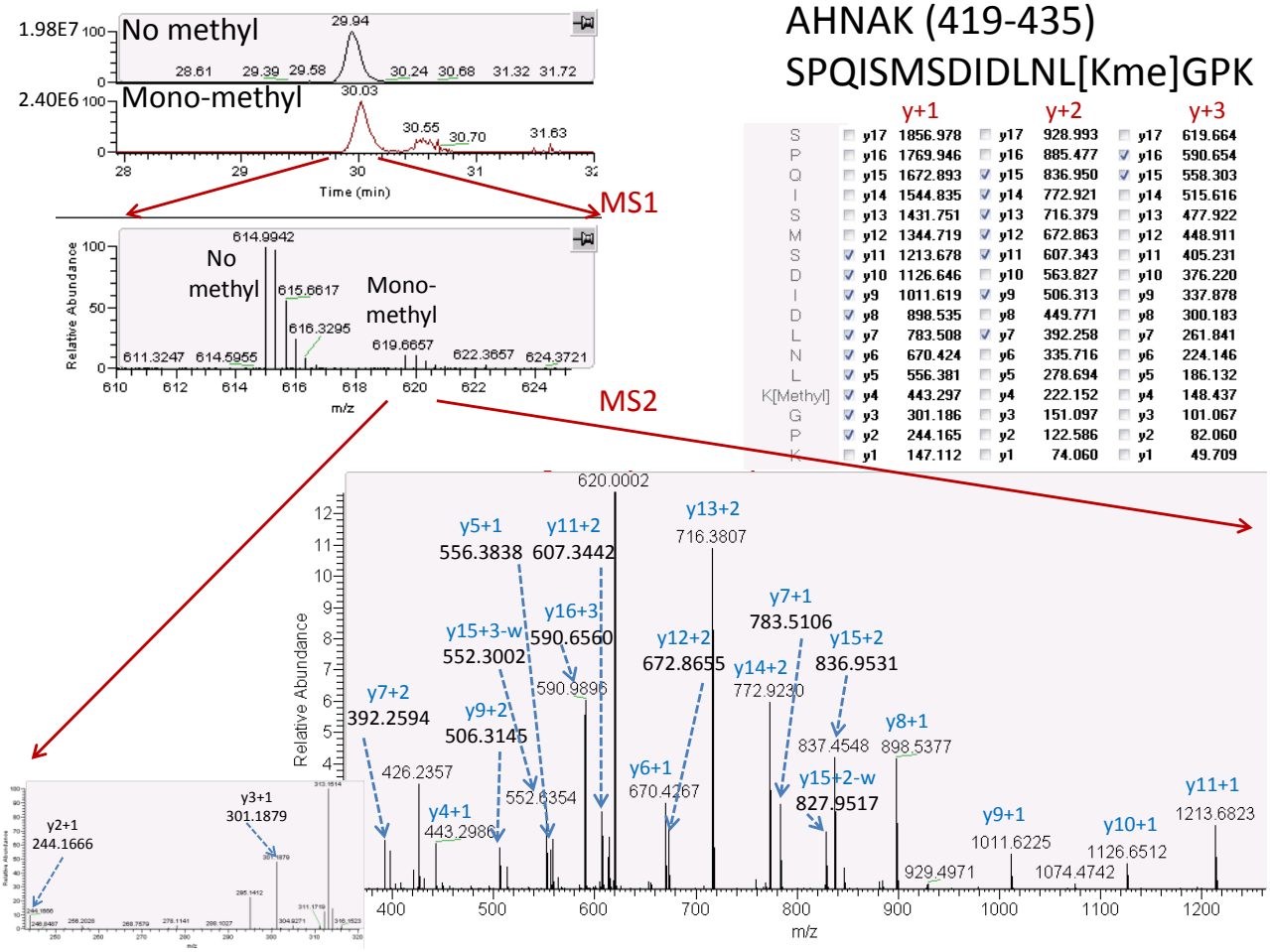


Figure S13: Peptide from recombinant AHNAK 4CRU protein (amino acids 419-435) with mono-methylation on K432 (equivalent to AHNAK-K4529me1 site in full-length protein). Both un-methylated and mono-methylated peptides are observed with the same amino acid sequence due to miscleavage at K432. Peptides with KGP motifs have a propensity to show high levels of miscleavage by trypsin in SILAC experiments. The two peptides have similar retention times, as shown in the extracted ion chromatograms (XICs) and MS1 spectrum in the top-left panel. The MS2 spectrum for methylated peptide is shown in the bottom part of the panel. The expected fragment m/z values are shown in the upper right panel for three different charge states of the fragment ions. Fragment m/z values with check marks were observed in the MS2 spectrum. The inset on the bottom left corner of the MS2 spectrum is a blow up of the low mass range showing y2 and y3 fragments as evidence for methylation specifically on K432, and not on the terminal K435.

Figure S13

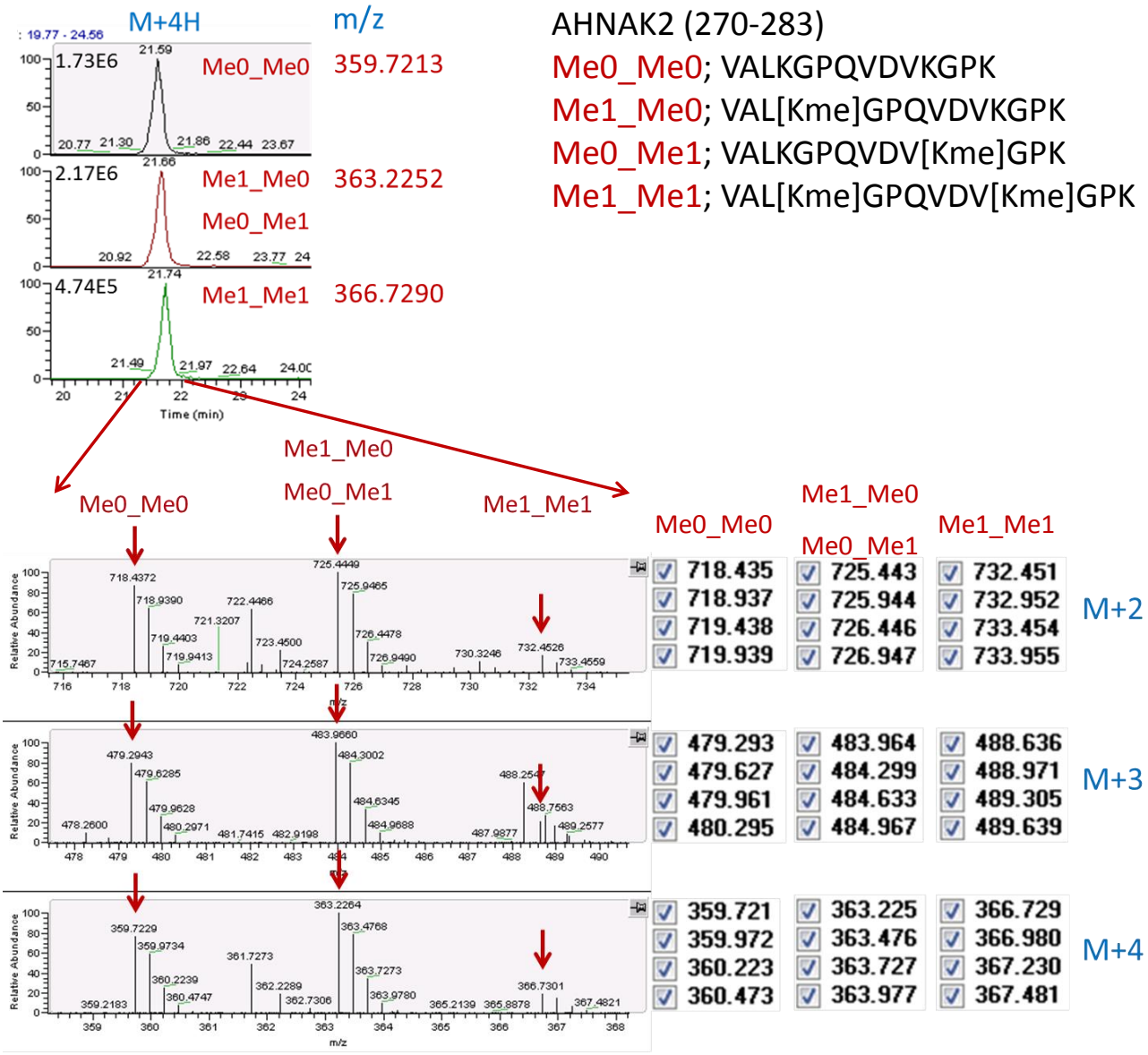


Figure S14A. MS1 spectra of the peptide from recombinant AHNAK2 4CRU protein (amino acids 270-283) with methylations on either K273 or K280 or on both. Peptide from recombinant AHNAK2 4CRU protein with methylation on either mono-methylations on either K273 or K280 or on both (equivalents to K1107me1 and K1114me1 sites in full-length protein). Upper left panel shows XICs for m/z = 359.7213, 363.2252, and 366.7290, corresponding to un-methylated, single mono-methylated, and double mono-methylated peptides, respectively, for the same amino acid sequence from AHNAK2 containing residues 270-283. Bottom left panel shows MS1 spectra of +2 (top), +3 (middle), and +4 (bottom) charge states of the peptides. All four major isotopes are clearly visible for all three charge states. On the bottom right, theoretical m/z values of the four major isotopes are shown as comparison. Similarly to the AHNAK (419-435) peptide, the sequence motif KGP shows significant miscleavage by trypsin even when there is no methylation on the Lys residue.

Figure S14A

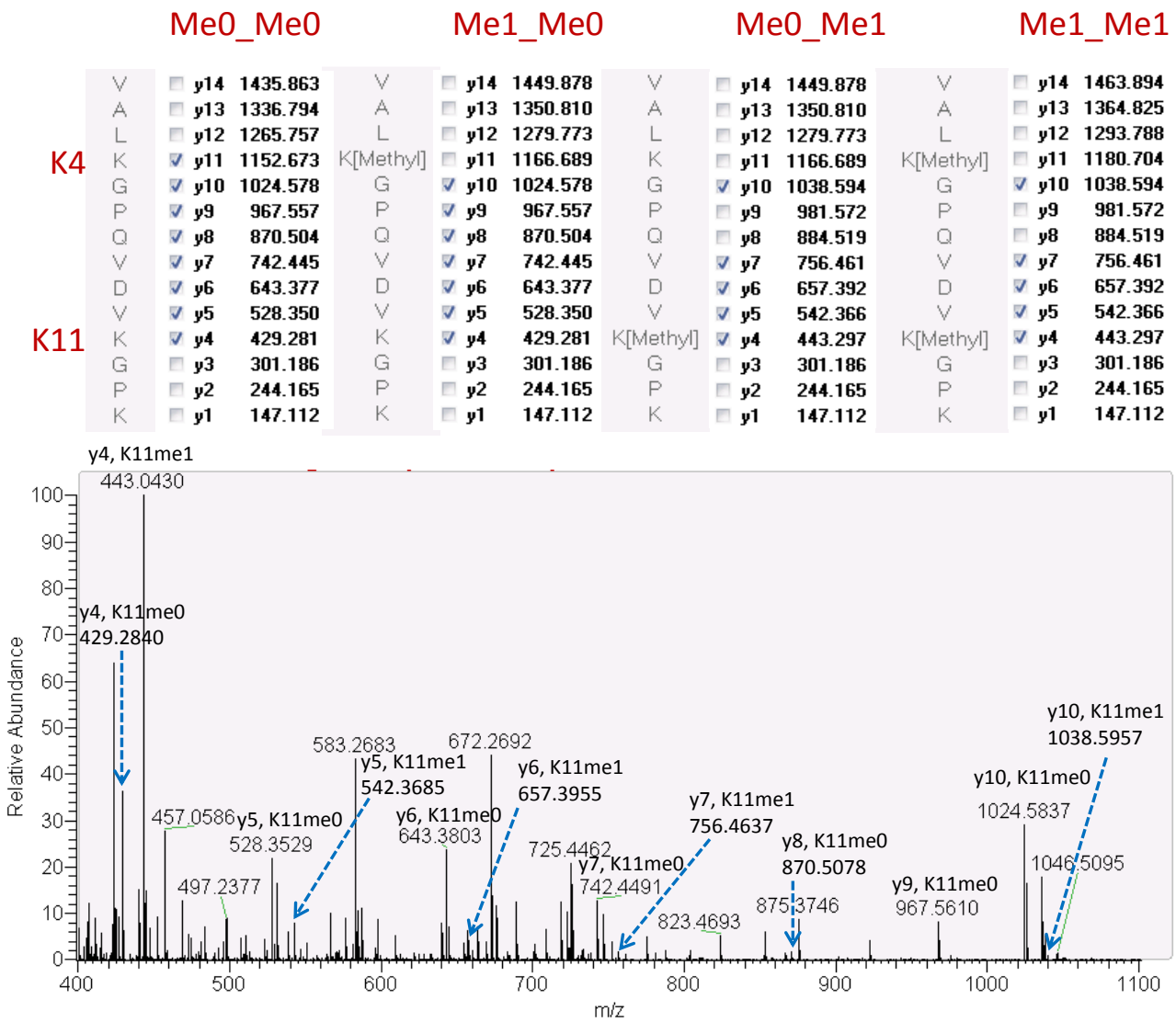


Figure S14B. Annotated MS2 spectra of the peptide from recombinant AHNAK2 4CRU protein (amino acids 270-283) depicting mono-methylation on K273, K280 or both (equivalent to K1107me1 and K1114me1 in full-length protein). MS2 spectra was obtained in All Ion Fragmentation (AIF) mode from the same sample that was used to collect MS1 data (Supplemental Figure S14A). The top panel shows the theoretical m/z values for y-ions for each of the possible methylation states. Check marks indicate which fragments were identified in the AIF-MS2 spectrum. Since MS2 spectrum was obtained in the AIF mode, the MS2 spectra alone does not necessarily prove or disprove the presence of a specific methylation state. However, the spectrum is consistent with co-migration of un-, mono-, and double mono-methylated peptides as shown by the high resolution MS1 data.

Figure S14B

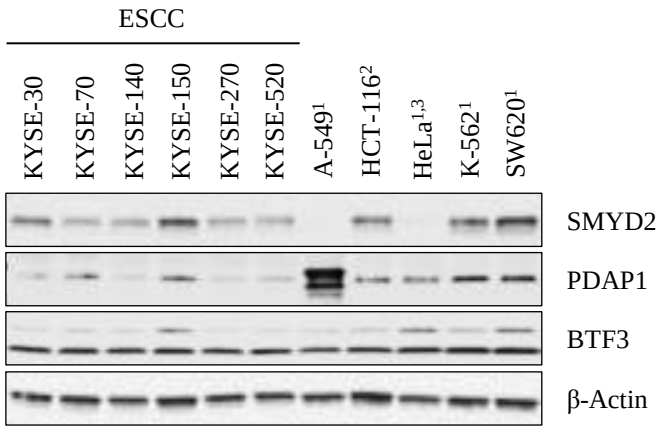


Figure S15. Protein expression of SMYD2 and SMYD2 substrates in mammalian cell lines. Western blot assays examining the protein levels of the indicated proteins across the indicated ESCC cell lines. Annotation refers to publication(s) reporting mono-methylated PDAP1 or BTF3. 1 = Wu *et al.*, 2014; 2 = Guo *et al.*, 2014; 3 = Cao *et al.*, 2012.

SUPPLEMENTARY METHODS

Correlation coefficients: Pair-wise uncentered correlation scores were calculated by using the SILAC ratios for all Kme1 sites in each biological replicate. Replicates were grouped according to correlation scores using averaged linkage hierarchical clustering.

siRNA knockdowns and Western blots: KYSE-150 cells were treated with ON-TARGETplus siRNA (Dharmacon, Lafayette, CO, USA) targeted against SMYD2 (L-020291-00-0005), BTF3 (L-016634-00-0005), PDAP1 (L-017675-00-0005), or control siRNA (D-001810-10) for 72 hours. Cell pellets were then lysed and run by SDS-PAGE and Western blot using the following antibodies accordingly to manufacturer recommendations: anti-SMYD2 (9734, Cell Signaling Technology), anti-BTF3 (ab203517, AbCam), anti-PDAP1 (4300, Cell Signaling Technology), anti-β-actin (A2228, Sigma-Aldrich). For biochemical methylation assays (Figure S13A), anti-SMYD2 (9734, Cell Signaling Technology), anti-p53 (2524, Cell Signaling Technology), anti-p53-K370me1 (generated in-house).

Immunofluorescence: KYSE-150 cells were treated with ON-TARGETplus siRNA (Dharmacon) targeted against SMYD2 (L-020291-00-0005) or AHNAK (L-014285-01-0005) for 72 hr, then fixed using 3.7% formaldehyde and permeabilized using 0.1% Triton X-100. Cells were incubated with anti-AHNAK antibody (ab68556, AbCam), followed by Hoechst (1µg/ml) and Alexa Fluoro 647 conjugate secondary antibody (A-21236, Invitrogen). Images were acquired by high-content imaging using Cell Insight NXT (Thermo Scientific).